Lipoproteins Inhibit the Secretion of Tissue Plasminogen Activator From Human Endothelial Cells

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Abstract We studied the effect of lipoprotein(a) [Lp(a)], low-density lipoprotein (LDL), and high-density lipoprotein (HDL) on tissue plasminogen activator (TPA) secretion from human endothelial cells. At 1 µmol/L, Lp(a) inhibited constitutive TPA secretion by 50% and phorbol myristate acetate- and histamine-enhanced TPA secretion by 40%. LDL and HDL also depressed TPA secretion by 45% and 35% (constitutive) and 40% to 60% (stimulated). TPA mRNA levels were also examined and found to change in parallel with antigen secretion. In contrast to TPA, plasminogen activator inhibitor type-1 secretion and mRNA levels were not affected by any of the three lipoproteins. These results suggest that the interaction of lipoproteins with certain cell-surface binding sites may interfere with the proper production and/or secretion of TPA.

Key Words • Lp(a) • tissue plasminogen activator • endothelial cells • plasminogen activator inhibitor type-1

At high levels, lipoprotein(a) [Lp(a)] is a major risk factor for premature development of atherosclerosis, myocardial infarction, and the severity of coronary artery disease. Low-density lipoprotein (LDL) is also atherogenic, but Lp(a) and LDL are independent risk factors for these diseases, suggesting a difference in their pathogenic mechanisms. Lp(a) is similar to LDL in composition and structural organization but contains an additional apoprotein, apo(a). Apo(a) is very similar in structure to plasminogen. It may contain 36 repeats of the fourth kringle of plasminogen as well as a single kringle 5 and a protease-like region. However, Lp(a) is not susceptible to cleavage by plasminogen activators. This similarity in structure between Lp(a) and plasminogen has led to the hypothesis that Lp(a) can interfere with the functions of plasminogen. In vitro studies of such effects have shown that Lp(a) inhibits plasminogen activation by streptokinase, suppresses the fibrin and cell surface-dependent enhancement of plasminogen activation by tissue plasminogen activator (TPA), and suppresses plasminogen binding to U937 monocytoid and endothelial cells. The LDL particle, which lacks apo(a), does not compete for plasminogen binding sites. Thus, Lp(a) interferes with plasminogen activation by inhibiting several molecular interactions of plasminogen.

In addition to the physical interference of Lp(a) with plasminogen binding to cells and other proteins, a prothrombotic state can be promoted by interference with the normal production and/or secretion of the fibrinolytic components associated with endothelial cells, ie, TPA, the plasminogen activator primarily responsible for blood clot lysis, and plasminogen activator inhibitor-1 (PAI-1). For example, inhibition of TPA release or elevation of PAI-1 production would create a prothrombotic state in the endothelial cell environment. It has been shown previously that addition of Lp(a) to human endothelial cells in culture stimulates PAI-1 production up to twofold over a 16-hour period. In the present study, we have examined the effects of Lp(a) on endothelial cell expression of the fibrinolytic components PAI-1 and TPA. We report here that the addition of Lp(a) to monolayers of human umbilical endothelial cells suppresses TPA secretion while having a minimal effect on PAI-1 secretion. However, similar results were also observed with LDL and high-density lipoprotein (HDL), suggesting a general mechanism by which lipoproteins might promote a prothrombotic state through the downregulation of TPA secretion without significantly affecting PAI-1 expression.

Materials

Phorbol myristate acetate (PMA) was from Calbiochem. Monoclonal antibodies to PAI-1 (No. II05) and TPA were from Monoclon and Corvas, Inc., respectively.

Cell Culture

All tissue culture reagents were purchased from sources previously described. Endothelial cells were isolated from human umbilical vein cord veins and were cultured into 75-cm² tissue culture flasks coated with 20 mg/mL calf skin gelatin. Cells were grown to confluence in RPMI 1640 containing 10% fetal calf serum, 200 U/mL penicillin, 200 µg/mL streptomycin, 10 µg/mL endothelial cell growth factor.
(EGF), and 90 μg/mL heparin. Passaged cells were subcultured into 12-well dishes or 75-cm² culture flasks and allowed to grow to confluence under the same conditions as primary cultures except that 50 μg/mL EGF and 15% fetal calf serum were used. All experiments used once-passaged cultures. Average cell densities at confluence were 6×10⁴ cells per square centimeter.

Studies were performed by washing confluent cultures twice with RPMI 1640 and incubating cultures at 37°C in 0.5 mL of medium containing 5% NuSerum (Collaborative Research; a final serum concentration of 1.25% newborn calf serum), 50 μg/mL EGF, 90 μg/mL heparin, and the indicated agent or vehicle. Stock solutions of phorbol esters (Calbiochem) were made in dimethyl sulfoxide (DMSO) (Calbiochem). In no case was the final concentration of DMSO added to cells greater than 0.1%. Vehicle concentration was kept constant in all experimental samples regardless of differences in the concentration of the compound tested. The presence of EGF, heparin, or DMSO had no effect on TPA release in the presence of any of the compounds used. When appropriate, conditioned medium was centrifuged at 15 000 g to remove cell debris, adjusted to 0.1% Tween 80, and frozen at −70°C until used.

Lipoprotein Isolation

LDL (d=1.019 to 1.063 g/mL) and HDL (d=1.063 to 1.21 g/mL) were isolated from 600 to 800 mL of pooled normal human plasma by ultracentrifugation in the presence of protease inhibitors as described. The isolated lipoproteins were dialyzed against 0.15 mol/L NaCl containing 272 μmol/L EDTA, pH 7.5, and their apoprotein composition was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Endotoxin contamination of the lipoproteins during isolation was reduced by the use of pyrogen-free sterile water, oven-baked glassware (185°C for 4 to 18 hours), and E-Toxa (-)-cleaned supplies. Endotoxin was monitored by the chromogenic Limulus amebocyte assay (Whittaker Bioproducts), which had a sensitivity of 0.1 endotoxin U/mL.

Lp(a) was isolated from fresh human plasma using flotation centrifugation followed by affinity chromatography on lysine Sepharose. The LDL preparation contained no apo-A-1, and the HDL contained no detectable apoB. Lipoprotein concentrations were determined by a modified Lowry assay. No contamination of the Lp(a) preparations was detected by immunoblot. These preparations were prepared from two different donors and consisted of a single isoform with an M₉ of 280 000.

Quantitation of TPA and PAI-1

TPA and PAI-1 antigens were quantified by enzyme-linked immunosorbent assay. Microtiter plates were coated with 10 μg/mL rabbit antiserum to human TPA or PAI-1, in 50 mmol/L sodium borate, pH 9.0, at 4°C overnight and washed with a wash buffer of 0.15 mol/L NaCl and 0.01 mol/L phosphate, pH 7.3 (PBS), containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA). For the TPA assays, samples or standards were diluted into culture medium, and 100 μL was added to the wells. Immediately afterward, 20 μL anti-human TPA monoclonal antibody horseradish peroxidase conjugate, diluted 1:50 into PBS/0.1% BSA, was added and the plate incubated for 1.5 hours at room temperature with rocking. The contents of the wells were removed, each plate was washed with water three times, and 100 μL of substrate was added. The substrate mixture was prepared by diluting 125 μg/mL trimethoxybenzoic acid into 0.1 mol/L sodium acetate, pH 4.5, containing 0.013% H₂O₂. The assays were allowed to develop for 10 minutes in the dark, and the reaction was stopped by the addition of 100 μL of 1N H₂SO₄. The plates were read at 450 nm. For PAI-1 measurements, assay samples and standards were diluted into PBS, 0.01% Tween 20, and 1% BSA, and 100-μL aliquots were incubated in the coated wells for 2 hours at room temperature. The plates were washed three times, and 0.25 μg/mL of monoclonal antibody to PAI-1, diluted into PBS/0.1% BSA/0.05% Tween 20 and 0.1% bovine γ-globulin, was added to the wells and incubated for 2 hours at 22°C. After washing, the wells were incubated with a 1:2500 dilution of horseradish peroxidase–conjugated rabbit anti-mouse IgG for 2 hours and then washed, and 1 mg/mL of 2′,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid in 0.0015% H₂O₂ was added. Plates were read at 405 nm after 45 minutes of incubation. Standard curves for both TPA and PAI-1 assays were determined by log-logit transformation and linear regression analysis of logit B (absorbance) versus log TPA or PAI-1 concentration. Values in the range of 0.2 to 12.5 ng/mL TPA and 1.6 to 25 ng/mL PAI-1 gave linear dose-response curves.

mRNA Quantitation

RNA was extracted by the method of Chomczynski and Sacchi, and TPA mRNA levels were measured by slot blot analysis as previously described. A full-length cDNA clone encoding the mature TPA protein plus 200 bp of the 5′-untranslated region and a PAI-1 cDNA clone containing the complete coding region, 80 bp of the 5′-untranslated region, and 140 bp of the 3′-untranslated region were used as probes for hybridization. An actin probe was used as a control (furnished by Dr L. Kedes, Stanford University).

Chromium-51 Labeling of Cells

Cells were labeled with 250 μCi/mL ¹¹⁵Cr (Amersham International) for 4 to 6 hours in medium containing 10% fetal calf serum. After the labeling period, cultures were washed twice with RPMI 1640 and then treated with medium containing 5% NuSerum and the indicated agonists in the presence or absence of lipoproteins for 16 hours. Medium was collected and cells were extracted with 0.5% Triton X-100. Both medium and cell extract were counted, and the chromium remaining was calculated as the percentage of cells incubated with agonist in the absence of lipoprotein.

Results

The effect of three different classes of lipoproteins, Lp(a), LDL, and HDL, on constitutive TPA secretion (12.2±4.3 ng/mL in 16 hours; range, 6.5 to 22.0 ng/mL, n=17) was examined by addition of increasing concentrations of each to confluent monolayers of endothelial cells. Lp(a) inhibited TPA secretion in a dose-dependent manner, with 1 μmol/L Lp(a) reducing TPA levels to 49.1±8.1% of control after 16 hours (Fig 1, top). Lp(a) was also effective against PMA-stimulated (100 nmol/L; Fig 1, bottom) TPA secretion. The level of TPA measured in the culture medium after 16 hours, a time when TPA levels reached their maximum levels in both cases, was reduced to 51.1±4.2% and 57±5.6%, respectively.

In contrast to the inhibitory effect of Lp(a) on plasminogen binding to cell surfaces, other lipoproteins were also effective at blocking both constitutive and stimulated TPA secretion. LDL and HDL inhibited constitutive release to 55.2±7.3% and 64.9±5% of control, respectively (Fig 1, top), histamine-stimulated TPA secretion to 49.7±7.9% and 67.9±1.5% (Fig 1, bottom), and PMA-stimulated secretion to 30.6±4.8% and 59.8±4.2% (Fig 1, middle), respectively. Addition of plasminogen (1 μmol/L) to the cells under identical conditions had no effect on TPA secretion (data not shown). The effect of LDL on various concentrations of histamine and PMA was also tested. At concentrations ranging from 0.1 to 20 μg/mL histamine, 1 μmol/L LDL inhibited the stimulated rate of TPA secretion by 30%
to 35% at each concentration. At concentrations of 10^{-10} to 10^{-7} \text{ mol/L} PMA, 0.5 \mu \text{mol/L} LDL inhibited secretion by 50% at each point.

PAI-1 antigen levels were also examined in replicate samples (Fig 2). Despite previous reports that Lp(a) stimulates PAI-1 secretion from endothelial cells,20 we detected no significant change in PAI-1 levels over the same period of time. To examine whether TPA mRNA levels are affected in the same manner as antigen levels, we performed slot blot analysis on cultures treated with vehicle, 1 \mu \text{mol/L} LDL, 100 \text{ nmol/L} PMA/100 \mu \text{mol/L} forskolin, and LDL/forskolin and LDL. Forskolin, which elevates cyclic AMP levels through the activation of adenylate cyclase, potentiates the PMA effect fivefold, making the quantitative measurement of TPA mRNA easier. Cyclic AMP does not, however, change any of the characteristics of the TPA response to PMA.26 Slot blot analysis of TPA mRNA showed a 67% decline in steady-state levels after 8 hours of LDL treatment (n = 2) (Fig 3). In the presence of LDL/forskolin, TPA mRNA increased 3.6-fold within the same time period but only 2.3 times in the presence of LDL. Whether the same effect on TPA mRNA is observed with HDL or Lp(a) is unknown. PAI-1 mRNA levels were unaffected by 1 \mu \text{mol/L} LDL (data not shown).

Discussion

We have demonstrated that the treatment of human umbilical vein endothelial cells with either Lp(a), LDL, or HDL results in a dose-dependent depression in both constitutive and stimulated TPA secretion. This effect on TPA secretion occurs with no significant change in the level of PAI-1 antigen over the same period of time.
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Fig. 3. Blots show effect of low-density lipoprotein (LDL) on tissue plasminogen activator (TPA) mRNA levels. Cultures were treated with or without 100 μmol/L phorbol myristate acetate (PMA) and 50 μmol/L forskolin (fors) for 8 hours in the presence or absence of 1 μmol/L LDL. Cells were extracted, and RNA was isolated as described in "Methods." TPA mRNA was measured by slot blot analysis; the values presented in the text were derived from comparing the counts per minute in the bands representing the LDL-treated samples with that of the respective control. Slots hybridized with a cDNA probe for actin are shown to demonstrate comparable loading of RNA.

Thus, abnormally high levels of Lp(a) and other lipoproteins may promote a prothrombotic state by manipulating the fibrinolytic capacity within the endothelial cell environment.

Our results contrast with the previous report of Etingin et al.,20 who found that Lp(a) enhanced PAI-1 antigen secretion from human endothelial cells in culture by twofold while having no effect on TPA production. It is not clear why this discrepancy exists, although the culture conditions under which these studies were performed were quite different. Etingin et al performed experiments in serum-free medium in contrast to the 1.25% serum used in the present studies. We have determined previously that the level of constitutive and stimulated TPA production and secretion in human umbilical endothelial cells is dependent on the presence of serum and that, in its absence, TPA secretion is unresponsive to modulating compounds.21 Another potential source for the differences in results may be related to the state of lipoprotein oxidation. For example, Latron et al27 and Kugiyama et al28 reported that oxidized but not nonoxidized LDL affects PAI-1 synthesis by endothelial cells. Our findings also are supported to some extent by in vivo observations.

In one study, patients with coronary artery disease and levels of Lp(a) 2.5 to 3.5 times higher than control subjects showed a decreased ability to release TPA in response to venous occlusion (50%), although basal levels of TPA were increased 76%. PAI-1 levels increased an average of only 25%. In the second study, a negative correlation between Lp(a) levels and PAI-1 levels was observed.30 Thus, our observation that TPA secretion can be inhibited by treatment of endothelial cells with lipoproteins is not unusual.

The mechanism by which the three lipoproteins inhibit TPA secretion is not clear, eg, whether it occurs through a protein or lipid moiety or whether it occurs through a general perturbation of the endothelial cell membrane or through a specific receptor or receptors. The lack of specificity of the lipoprotein effect shown in Fig 1 suggests that it is the lipid components of the lipoproteins that are responsible for the biologic activity, because the various lipoproteins do not contain common apoproteins but do contain various common lipid components, although at different relative proportions. A number of the lipid constituents are viable candidates, including the lysolcithins, oxygenated steroids, and free fatty acids. The fact that all three lipoproteins have similar effects also suggests that the site of interaction is similar in each case, a suggestion consistent with recent observations that LDL and HDL can both inhibit Lp(a) binding to endothelial cells and that gangliosides, which inhibit plasminogen binding to endothelial cells, also inhibit both Lp(a) and LDL binding.33 Although HDL and LDL generally exert counterbalancing effects, competition between HDL and LDL for cellular binding sites on endothelial cells and other cells has been noted. Tabas and Tall36 suggested that the association of HDL with endothelial cells may be dependent on lipid interactions that could be shared by all lipoprotein classes. Lipid transfer mechanisms28 may also be involved and could be shared among the various lipoproteins. The interaction of lipoproteins with certain shared cell surface binding sites such as lipids may interfere with the proper production and/or secretion of TPA.

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References


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